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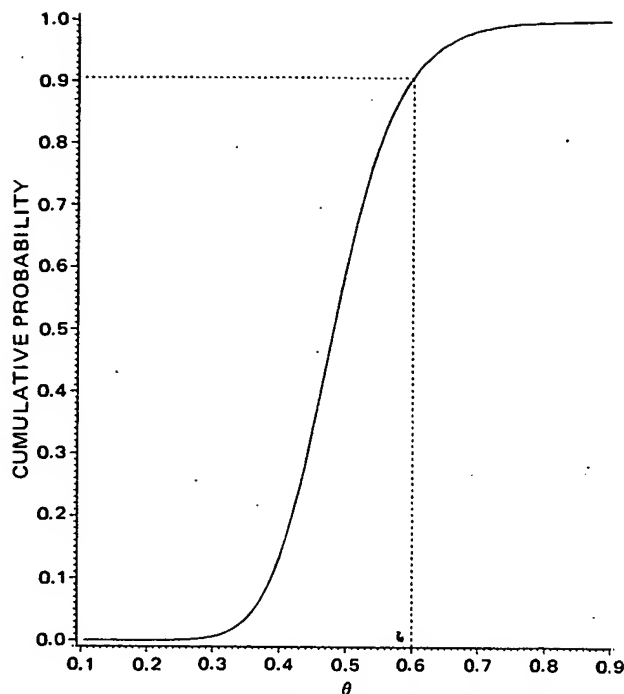


Figure 4—Cumulative distribution function for $\theta = (C_{\max})_{\text{NEW}} / (C_{\max})_{\text{STD}}$.

be of interest to know the probability that θ is >0.8 . This may be read from Fig. 3 and is 0.866—a probability which still might not be high enough to state bioequivalence. In addition it might be desirable to know the probability of θ being >0.7 , which also may be read from Fig. 3 and is 0.98.

3. The assessment with respect to C_{\max} , that θ is <0.6 (Eq. 12) yielded a posterior probability of 0.906. Similarly it might be desirable to know the probability of θ being >0.7 . This probability can be read from Fig. 4 and is 0.02.

4. The method of symmetrical confidence limits proposed by Westlake (1) causes the loss of information since it gives the false impression that the ratio is symmetric about 1. As Mandallaz and Mau (3) have shown, the symmetric confidence interval approach may give exactly the same 95% confidence intervals for two sets of data while having completely different posterior distributions for θ , because of the differing variances and locations of the posterior distributions.

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In Vivo and In Vitro Release of Macromolecules from Polymeric Drug Delivery Systems

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Received January 26, 1982, from the *Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139 and ‡Department of Surgery, Children's Hospital Medical Center, Boston, MA 02115. Accepted for publication August 30, 1982.

Abstract □ *In vivo* release rates of a macromolecule from an ethylene-vinyl acetate copolymer have been shown to be indistinguishable from those of identical implants tested *in vitro*. The studies were conducted for ~2 months, and two different techniques were used to assess release rates. One of these techniques, using [³H]inulin as a marker, may be particularly useful in future studies assessing *in vivo* release rates from drug delivery systems. The appearance of [³H]inulin in the urine of rats bearing implants allowed continuous monitoring of release. A histological evaluation of tissue sections surrounding polymer implanted for 7 months showed no inflammatory cell reaction.

Keyphrases □ Drug delivery systems—ethylene-vinyl acetate copolymer matrix, inulin, release kinetics, *in vitro-in vivo* comparison □ Ethylene-vinyl acetate copolymer—sustained release of inulin, release kinetics, *in vitro-in vivo* comparison □ Inulin—sustained release using polymeric matrices, release kinetics, *in vitro-in vivo* comparison

Since the first report that biocompatible polymers such as ethylene-vinyl acetate copolymer could be used for the controlled release of macromolecules (mol. wt. > 1000) (1), these systems have been used by different investigators in biological (2–11), ophthalmological (12–17), neurological (18, 19), and microbiological research (20, 21). Macromolecules such as enzymes (22), antigens (23), and insulin (24) have been released in biologically active form for up to 6 months *in vivo*. Extensive studies *in vitro* have demonstrated that the release rates of drugs from these devices

can be adjusted over a 2000-fold range by simple alterations in the fabrication procedures of the macromolecule-polymer matrices (25).

The macromolecules incorporated into these polymer matrices are usually proteins. Thus, once released *in vivo*, they are degraded to amino acids and recycled to other body proteins. Neither the native proteins nor their metabolites are excreted. For this reason, it has been difficult to directly measure the absolute release rates of such macromolecules *in vivo*. We now report two new methods to measure *in vivo* release which demonstrate that release kinetics from ethylene-vinyl acetate copolymer implants *in vivo* are indistinguishable from identical implants tested *in vitro*. In one method, release rates of ¹⁴C-labeled proteins were determined by assaying the remaining radio-labeled protein in the implants at various time points *in vivo* and *in vitro*. In the second method, the use of the polysaccharide inulin, which is totally excreted (26, 27), permitted direct *in vivo* monitoring of release kinetics by collecting urine and assaying for inulin. These studies should enable investigators to employ ethylene-vinyl acetate copolymer matrices with the knowledge that predetermined *in vitro* release kinetics will be followed *in vivo*.

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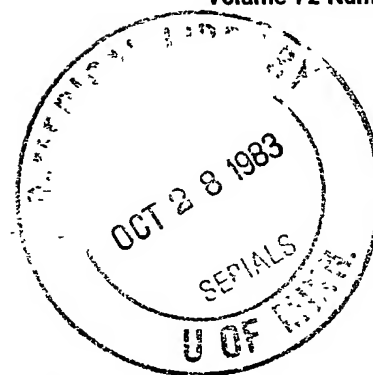
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EXPERIMENTAL

Isotope Preparation—Bovine serum albumin¹ (12.2 g) was dissolved in 100 ml of double glass-distilled water. [¹⁴C]Methyl bovine serum albumin² (1 μ Ci) was added, and the solution was stirred for 1 hr at 23° with a magnetic stirrer³. The solution was then lyophilized⁴ yielding a final specific activity for the [¹⁴C]methyl bovine serum albumin of 0.082 nCi/mg. β -Lactoglobulin A¹ (6.02 g) was dissolved in 100 ml of double glass-distilled water, and 5 μ Ci of [¹⁴C]methyl β -lactoglobulin A² was added. The solution was then lyophilized yielding a final specific activity for [¹⁴C]methyl β -lactoglobulin A of 0.83 nCi/mg. Inulin¹ (1.43 g) was suspended in 50 ml of reagent-grade acetone⁵ in an Erlenmeyer flask. [³H]Inulin² (5 mCi) was added, and the suspension was stirred for 1 hr at 23° with a magnetic stirrer. The suspension was collected on filter paper⁶ in a Büchner funnel, rinsing the flask with acetone to remove residual [³H]inulin. The [³H]inulin was collected and dried overnight in a desiccator; the resulting specific activity was 3.44 μ Ci/mg.

Matrix Preparation—Ethylene-vinyl acetate copolymer⁷ was rinsed with water and alcohol to remove inflammatory impurities (2) and dissolved in methylene chloride. The three radioactive macromolecule powders were pulverized with a spatula to produce a normal distribution of particle sizes. As a safety precaution, the powders were not sieved to varying particle size ranges as is often done (25), due to the aerosol effect of shaking radioactive powders.

Fifteen milliliters of 10% (w/v) polymer solution was added to each of six glass vials⁸ containing 807 \pm 3.1 mg of the pulverized [¹⁴C]methyl bovine serum albumin. The polymer-protein mixture was poured into a rectangular mold precooled to -80° and then freeze-dried, at -20° (25). The resulting matrices were 35% (w/w) loaded with [¹⁴C]methyl bovine serum albumin. Sixty-seven 1 cm \times 1-cm squares were excised from the six slabs (25) with a surgical scalpel blade⁹. The mean weight and standard deviation of the 67 matrices were 67.9 \pm 2.9 mg.

Fifteen milliliters of 10% (w/v) polymer solution was added to each of five glass vials containing 989.0 \pm 74.0 mg of pulverized [¹⁴C]methyl β -lactoglobulin A. The procedure for matrix formation was followed as above, yielding five 40% loaded (w/w) protein polymer matrix slabs. Sixty-seven 0.8 cm \times 0.8-cm squares were excised from the five slabs. The mean weight and standard deviation of the 67 matrices were 44.5 \pm 1.2 mg.

Fifteen milliliters of 5% (w/v) polymer solution was added to each of three glass vials containing 568 \pm 25.4 mg of [³H]inulin. Three 44%-loaded (w/w) [³H]inulin polymer matrices were formed. Twenty-nine 1 cm \times 1-cm squares were excised from the three slabs. The mean weight and standard deviation of the 29 matrices were 82.00 \pm 8.0 mg.

Release of Macromolecules—Kinetic studies, both *in vitro* and *in vivo*, were conducted on the release of three radioactively labeled macromolecules: [¹⁴C]methyl bovine serum albumin (mol. wt. 68,000), [¹⁴C]methyl β -lactoglobulin A (mol. wt. 18,000), and [³H]inulin (mol. wt. 5200). For the *in vitro* studies, 30 of each set of the ¹⁴C-labeled protein-polymer squares and 12 [³H]inulin-polymer squares were placed in glass vials containing 5 ml of sterile phosphate-buffered saline¹⁰ (pH 7.4) and 0.1% sodium azide⁵ (release media). The vials were placed on a shaker¹¹ at 37°. At various time points during the experiment, five polymer squares were removed from the release experiment for the determination of unreleased ¹⁴C-labeled protein in the polymer matrices using an isotope recovery method (see below). The remaining polymer squares were transferred to fresh release media to continue the *in vitro* release.

For the *in vivo* studies, 30 ¹⁴C-labeled protein-polymer squares identical to those used *in vitro* were implanted into 100- to 150-mg male CD rats¹². The polymer squares were implanted by making a 2-cm incision in the lower abdominal area of the rat with a scalpel. A pair of sterile round-edged scissors was then used to create a pocket in the subcutaneous tissue ~5 cm from the incision. The polymer square was placed into the pocket with sterile forceps. The wound was closed with animal wound clips¹³. Five polymer squares were removed at the same time points as

in the *in vitro* experiments for the determination of unreleased ¹⁴C-labeled protein in the polymer matrices.

Thirteen [³H]inulin polymer matrices were implanted into 100- to 150-g male CD rats in the manner described above. The rats were housed in metabolism cages¹⁴ for the duration of the experiment. Urine was collected daily in 15-ml graduated collection tubes¹⁵. The urine volumes were read directly off the graduated urine collection tubes.

Release Kinetics—Isotope Recovery Method—Release rates for the ¹⁴C-labeled protein experiments were determined by assaying the remaining radiolabeled bovine serum albumin or β -lactoglobulin A in each polymer square. At various time points during the experiment, five polymer squares were removed from both the rats and the release media. They were placed in scintillation vials, lyophilized to remove residual water, and dissolved in 1 ml of xylene¹⁶. When the polymer dissolved, the unreleased macromolecule precipitated to the bottom of the vial. One milliliter of distilled water was added to the vial to dissolve the precipitate. Ten milliliters of xylene-based scintillation fluor¹⁷ was added to the dissolved precipitate, and the radioactivity was measured by liquid scintillation counting¹⁸. The radioactivity remaining in the matrix at any given time was subtracted from the average amount of radioactivity remaining at the previous time point; release rates are expressed as dpm/hr. The procedure described above was used to dissolve the polymer and to precipitate unreleased [³H]inulin in each of two polymer squares at each time point. Then, 10 ml of warm distilled water (70–80°) was added to the dissolved [³H]inulin-polymer matrix to solubilize the inulin precipitate. A 200- μ l aliquot of water was then removed and counted¹⁸.

Before the start of the ¹⁴C-labeled protein release experiments, 7 of the 67 polymer squares were assayed by the above method to establish the initial dpm per polymer matrix. In a similar manner 4 of the 29 [³H]inulin-polymer squares were assayed to determine the mean dpm per polymer matrix.

Isotope Release Method—In the *in vitro* studies, the amount of radiolabeled macromolecule released was determined by removing a 250- μ l aliquot from the release media, adding 4 ml of scintillation fluor, and counting the solution¹⁸. For the *in vivo* studies [³H]inulin-polymer matrix squares were implanted into five rats housed in metabolism cages. Urine was collected and measured daily. A 250- μ l aliquot of urine was added to 4 ml of scintillation fluor plus 400 μ l of distilled water in a glass miniscintillation vial¹⁹ and the solution was counted¹⁸. The channels ratio method of quench correction was used to determine the counting efficiency of the urine samples (28).

Statistics—*In vivo* and *in vitro* release rates were compared by an analysis of covariance on a log transformation of the release rate data (29). The natural logarithms of the release rates and times were calculated. This transformation resulted in a linear relationship for each data set with r^2 ranging from 0.89 to 0.96 for the regression lines. The *in vivo* and *in vitro* regression lines were then compared as follows: The mean square error from the regression model with a separate slope and intercept for each trial condition (there were three trial conditions for bovine serum albumin, three trial conditions for β -lactoglobulin A, and two trial conditions for inulin) was compared with the mean square error for the regression model with common slopes and intercepts for all trial conditions pertaining to a specific macromolecule. The resulting test statistic was compared to the F distribution (30). The regression lines were considered indistinguishable if $p > 0.05$.

Histology—Histology was performed on tissues surrounding washed (2) ethylene-vinyl acetate copolymer subcutaneous implants containing bovine serum albumin after 7 months. The polymer matrices were implanted into male CD rats. An unwashed industrial grade ethylene-vinyl acetate copolymer⁷ was used as a control to exhibit an inflammatory response. Sections (5 μ m) were prepared using hematoxylin and eosin staining.

RESULTS

Comparison of Release Rates of ¹⁴C-Labeled Proteins *In Vitro* and *In Vivo*—Figure 1 compares the release rates of the 35% loaded (w/w) [¹⁴C]methyl bovine serum albumin-polymer matrices *in vivo* and *in vitro* as measured by the recovery of unreleased protein in the polymer and as measured directly in the *in vitro* release media. Throughout the

¹ Sigma Chemical Co., St. Louis, Mo.

² New England Nuclear, Boston, Mass.

³ Model PC-35 magnetic stirrer; Corning Glass Works, Corning, N.Y.

⁴ Virtis #10-148 lyophilizer; The Virtis Co., Gardiner, N.Y.

⁵ Fisher Scientific Co., Fair Lawn, N.J.

⁶ Grade 595 filter paper; Schleicher and Schnell, Keene, N.H.

⁷ Elvax 40P; Dupont Chemical Co., Wilmington, Del.

⁸ Wheaton Scientific Co., Millville, N.J.

⁹ No. 10 blade; Bard-Parker, Rutherford, N.J.

¹⁰ Grand Island Biological Co., Grand Island, N.Y.

¹¹ Clinical Rotating Apparatus, set at speed 3; Arthur H. Thomas, Philadelphia, Pa.

¹² Charles River Breeding Laboratories, Wilmington, Mass.

¹³ Autoclip Kit; Clay Adams, Parsippany, N.J.

¹⁴ Econo Metabolism Unit; Scientific Products, McGraw Park, Ill.

¹⁵ No. 2095 graduated centrifuge tube; Falcon, Oxnard, Calif.

¹⁶ Mallinckrodt Inc., Paris, Ky.

¹⁷ Aquasol 2; New England Nuclear, Boston, Mass.

¹⁸ Packard Model 3320 Liquid Scintillation Counter; Packard Instruments, Co., Inc., Downers Grove, Ill.

¹⁹ Rochester Scientific, Rochester, N.Y.

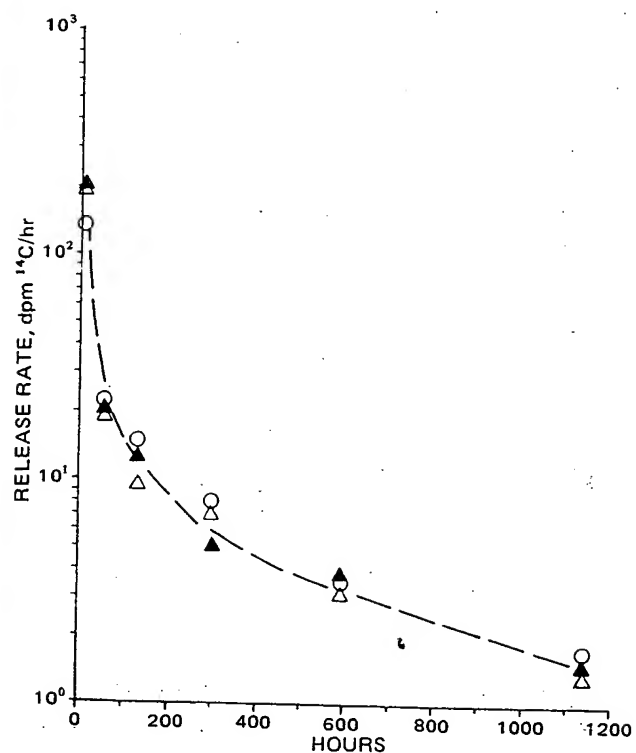


Figure 1—In vivo and in vitro comparison of release rates from 35% loaded (w/w) [^{14}C]methyl bovine serum albumin polymer matrices. Each point represents the average release of five polymer squares. Key: (Δ) in vivo recovery; (\triangle) in vitro recovery; (\circ) in vitro release.

experiment, *in vivo* and *in vitro* release rates were not statistically different ($p > 0.05$). Figure 2 shows that the release rates of 40% loaded (w/w) [^{14}C]methyl β -lactoglobulin A-polymer matrices *in vivo* and *in vitro* also were not statistically different ($p > 0.05$). At each time point

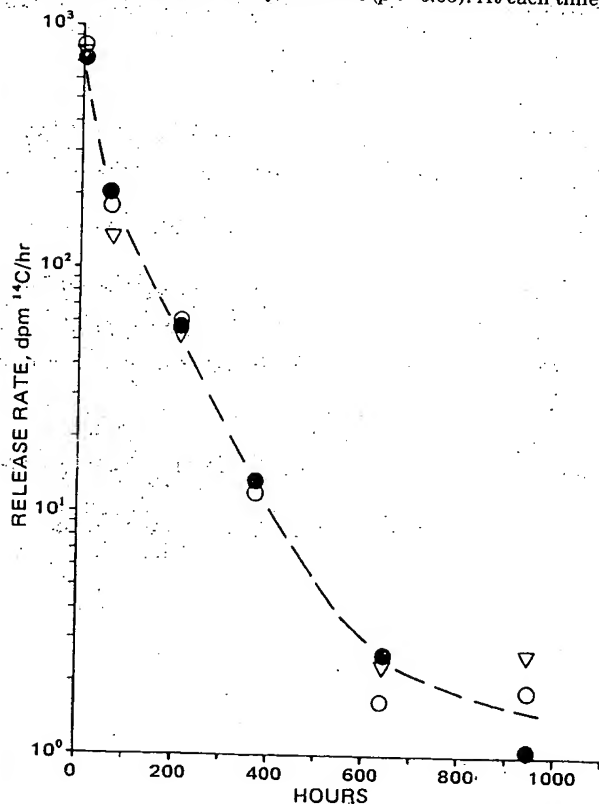


Figure 2—In vivo and in vitro comparison of release rates from 40% loaded (w/w) [^{14}C]methyl β -lactoglobulin A polymer matrices. Each point represents the average of five polymer squares. Key: (\circ) in vitro recovery; (\bullet) in vitro release; (∇) in vivo recovery.

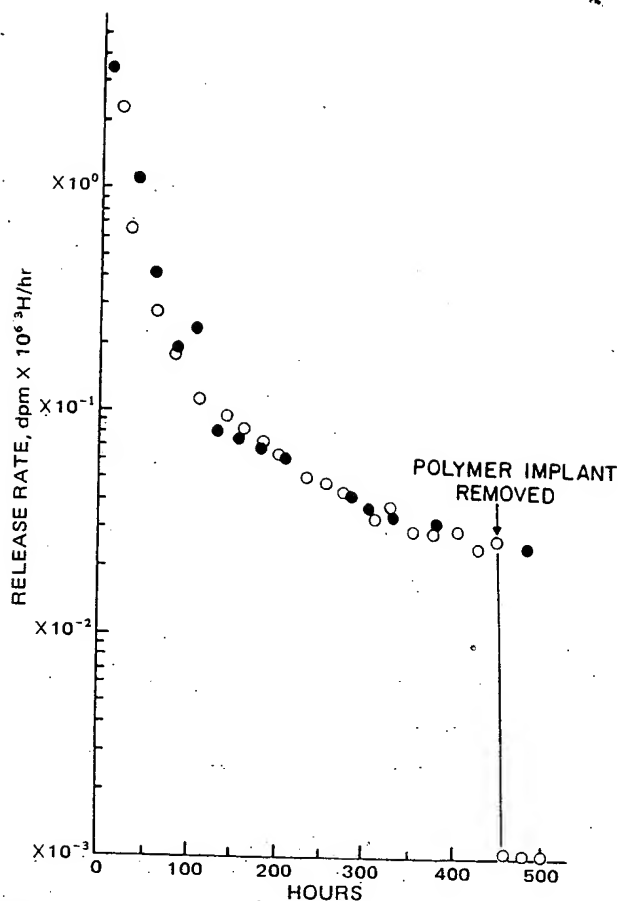


Figure 3—In vivo and in vitro comparison of release rates from 44% loaded (w/w) [^3H]inulin. Each *in vivo* point (\circ) represents the average release rate obtained by the collection of urine from five rats. Each *in vitro* point (\bullet) represents the average release rate from four polymer squares into phosphate-buffered saline. The polymer squares were removed from the rats after 450 hr.

the recovered and released radioactivity were totaled for each polymer square. Complete material balance was demonstrated as this total equaled ($\pm 7\%$) the initial amount of radioactivity incorporated into each polymer square. The material balance can also be seen by the equivalent *in vitro* release rates obtained by both the recovery and release methods (Figs. 1 and 2).

Comparison of [^3H]Inulin Release *In Vivo* and *In Vitro*—Figure 3 compares the release rates of [^3H]inulin analyzed directly by the collection of urine from five implanted rats and four polymer squares releasing *in vitro*. Release rates *in vivo* and *in vitro* were indistinguishable ($p > 0.05$). As an internal control, the polymer implants were removed from the rats at 450 hr into the experiment. The rats were placed in the metabolism cages for an additional 50 hr. Within 4.5 hr after the explant of the polymer, recovery of [^3H]inulin dropped 51-fold as measured in the urine of the animals. Release rates decreased for the remainder of the experiment (Fig. 3). An additional experiment was conducted over a 1500-hr period with a smaller sample size and showed an *in vitro-in vivo* relationship similar to the above (data not shown).

The possibility of artifacts in the amount of inulin recovered due to tritium exchange with surrounding aqueous media was checked by lyophilizing a known amount of [^3H]inulin. If exchange did occur, the water removed during the lyophilization procedure would cause a reduction in the dpm in the sample. No such reduction in the amount of radioactivity was observed, confirming that tritium exchange did not occur.

In a separate experiment, the recovery of unreleased [^3H]inulin from polymer matrices releasing *in vivo* and *in vitro* was compared at five different time points over a 30-day period. The amounts of [^3H]inulin recovered at various times in both cases were not statistically different ($p > 0.05$).

Histology—Figure 4A shows the tissue response to the washed (2) polymer implants containing bovine serum albumin which were removed after 7 months. The polymer implant (P) was surrounded by a very thin capsule of connective tissue. There was no inflammatory reaction, and the

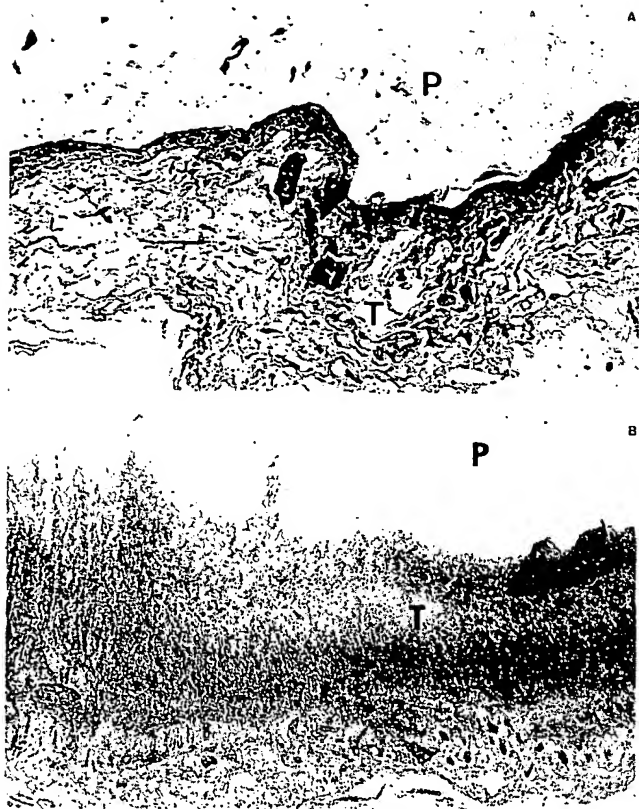


Figure 4—(A) The washed implant (P) is surrounded by a thin capsule of dense connective tissue (T). There is virtually no inflammatory reaction, and the adjacent loose connective tissue is normal. Original magnification 100X. **(B)** The tissue (T) surrounding the control unwashed implant (P) shows an extensive inflammatory reaction. The granulation tissue is thick and shows multiple layers, some infiltrated with abundant polymorphonuclear leukocytes. Original magnification 100X.

adjacent loose connective tissue was normal. As a control, unwashed polymer resulted in tissue sections that showed an extensive inflammatory reaction. The granulation tissue surrounding the control polymer was thick and showed multiple layers, some infiltrated with abundant polymorphonuclear leukocytes (Fig. 4B).

DISCUSSION

These results demonstrate that *in vivo* release kinetics of a particular macromolecule from ethylene-vinyl acetate copolymer matrices closely follow *in vitro* release kinetics of identical implants under physiological conditions. These studies were carried out for three macromolecules with a wide range of molecular weights (5200–68,000) and loadings (35–44%).

Many macromolecules incorporated into the ethylene-vinyl acetate copolymer matrix are proteins or other biological substances (2–25) that are metabolized and redistributed into other body tissues. In these cases, *in vivo* release rates cannot be measured directly. To circumvent this problem, a procedure had to be developed for the recovery of radioactively labeled macromolecules from the ethylene-vinyl acetate copolymer matrix. The implants were removed from the experimental animals, or from the release media in the *in vitro* studies. The squares were first lyophilized to improve the solubility of the polymer in xylene. Xylene was the solvent of choice for two reasons:

1. On dissolution of the polymer, the protein remaining in the polymer precipitated to the bottom of the glass vial. The addition of water to dissolve the precipitated protein was facilitated by the greater density of water compared with xylene. Thus, the ethylene-vinyl acetate copolymer in xylene floated on the upper organic phase, while the water dissolved the precipitated protein in the bottom aqueous phase.

2. The scintillation fluor used to count the radioactivity in the dissolved polymer was a xylene-based fluor. Thus, it was completely compatible with both the xylene-ethylene-vinyl acetate copolymer phase and the aqueous phase. This resulted in a homogeneous translucent

emulsion, suitable for liquid scintillation counting. Control studies demonstrated that there was no quenching due to the polymer in the scintillation fluor.

Using this technique, *in vivo* release rates of ^{14}C -labeled proteins were shown to be statistically indistinguishable from *in vitro* release rates with identical implants (Figs. 1 and 2). However, this technique had several disadvantages: (a) the need for a large sample size of polymer matrices and animals (30 rats were used in each experiment), (b) the use of long half-life ^{14}C -labeled proteins, and (c) the inability to directly measure release rates from polymer implants *in vivo*.

In contrast to the proteins, the polysaccharide inulin (mol. wt. 5200) is not metabolized *in vivo* nor reabsorbed by the kidney tubules. Once absorbed into the bloodstream, inulin is totally excreted in the urine (26, 27). Thus, inulin seemed to be an ideal model drug with which to measure *in vivo* release directly by simply collecting urine from ^3H -inulin-polymer-implanted rats housed in metabolism cages. Figure 3 clearly demonstrated that *in vivo* and *in vitro* release rates of ^3H -inulin are indistinguishable. The value of using inulin as a model drug to compare *in vivo* and *in vitro* macromolecule release was shown by its nearly instantaneous appearance in the urine after polymer implantation. When the polymer implant was removed after 450 hr (Fig. 3), release rates dropped 51-fold within 4.5 hr. This was further evidence that inulin release was only associated with the presence of the polymer implant. In addition, the use of ^3H -inulin allowed the continuous monitoring of release *in vivo*. The use of five rats in this experiment versus the 30 rats needed for the ^{14}C -labeled protein recovery experiments provided for a simpler experiment design.

The continuous decrease in release rates of macromolecules from the ethylene-vinyl acetate copolymer matrix is predictable from the flat-slab geometry used in these experiments (31). It has been suggested that release occurs through a porous network of tortuous channels created by the powdered drug during the fabrication procedure of the polymer matrix (32). Thus, drug release rates decrease due to increasing diffusion distance through the pores of the matrix as time increases. Constant release rates have been obtained with appropriate geometric design (33). The relationship of *in vivo* and *in vitro* release rates are now being studied for these alternative geometries.

The excellent biocompatibility of washed ethylene-vinyl acetate copolymer was demonstrated by a 7-month polymer implant into subcutaneous rat tissue (Fig. 4). This is consistent with previous studies in which this polymer was shown to be inert in rabbit corneal implants (34). It is conceivable that other polymers may either be subject to bioerosion or induce significant fibrous encapsulation that could cause *in vivo* and *in vitro* release rates to differ. Thus, ethylene-vinyl acetate copolymer may prove to be a useful standard for judging other polymers with respect to biocompatibility and *in vivo* release kinetics.

The results presented here demonstrate that *in vivo* release kinetics are statistically indistinguishable from *in vitro* release kinetics using identical implants for macromolecules with a wide range of molecular weights and loadings. The implication is that a desired *in vivo* release rate could be manipulated through *in vitro* testing. This is of particular importance if these polymer matrices are to be used experimentally (24) or clinically for the delivery of macromolecules such as insulin. Furthermore, the inulin-polymer model establishes a methodology that can be easily applied for *in vivo-in vitro* comparisons for any controlled-release system. Tracer amounts of inulin could be mixed with any drug to monitor the continual effectiveness of an implant in animals or humans. Small implantable sustained-release inulin-polymer matrices may also be useful for extended studies of glomerular filtration. These data show that *in vivo* release can be accounted for by the same mechanisms operating *in vitro*; this should now make possible the further development and increased use of ethylene-vinyl acetate copolymer drug delivery systems.

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Absorption of Triazolam from Pelleted Drug-Diet Mixtures by the Mouse: Quantitation of α -Hydroxytriazolam in Urine

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Abstract □ The absorption of triazolam from pelleted drug-diet mixtures by mice under steady-state conditions was determined for doses up to 150 mg/kg/day by measuring α -hydroxytriazolam, the principal urinary metabolite of triazolam in the mouse, in urine samples collected over a 24-hour period. Following β -glucuronide glucuronosohydrolase hydrolysis of the urine, quantitation of α -hydroxytriazolam was accomplished using a specific reverse-phase liquid chromatographic method which utilized UV detection at 214 nm. Assay precision was $>2.7\%$ (CV) over the concentration range of interest. Statistical analysis of the excretion data indicated that the mathematical relationship between the triazolam dose and the quantity of α -hydroxytriazolam excreted was linear for female mice and nonlinear for male mice. Triazolam absorption, as reflected by α -hydroxytriazolam urinary excretion data, increased with triazolam dose.

Keyphrases □ Triazolam—absorption from drug-diet mixtures by mice, determination by metabolite excretion in urine, α -hydroxytriazolam □ α -Hydroxytriazolam—urinary excretion, use to measure triazolam absorption in mice, drug-diet mixtures □ Absorption—triazolam in mice, drug-diet mixtures, measurement by α -hydroxytriazolam excretion in urine

The incorporation of drugs into the laboratory diet of mice and rats is a convenient and commonly used method

of administering drugs in chronic toxicology studies since it eliminates the need for time-consuming daily administration of aqueous solutions or suspensions by gavage. In addition to its convenience, this mode of administration also eliminates the daily trauma and danger of pulmonary complications associated with dosing by gavage. As is well known, however, the administration of a drug in a carrier, such as laboratory diet, may affect drug absorption. Information concerning the absorption of a drug from the carrier over the range of dosages administered in toxicological studies may be useful, if not essential, in assessing the significance of study results. It should also be noted that the Food and Drug Administration's recently instituted Good Laboratory Practice regulations may require that the degree of absorption of a drug from a carrier be determined (1).

Several methods have been reported for determining the relative absorption of drugs following administration of pulverized drug-diet mixtures. Van Harken and Hottendorf (2) determined the exposure of rats to cefatrizine, under steady-state conditions, by comparing the area